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SYMPOSIUM: RESEARCH ADVANCES AFTER A DECADE OF WAR

Rifamycin Derivatives Are Effective Against Staphylococcal Biofilms In Vitro and Elutable From PMMA

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Abstract

Background Local antimicrobial delivery through polymethylmethacrylate beads (PMMA), commonly vancomycin, is used for the treatment of contaminated open fractures but has limited activity against *Staphylococcus aureus* biofilms, which occur commonly in such fractures. Rifamycins have activity against biofilms and are an effective treatment for osteoarticular infections involving staphylococcal biofilms, but there are limited studies evaluating the activity of rifamycin derivatives, other than rifampin, against biofilms of *S. aureus* and evaluating incorporation of these drugs into PMMA for treatment of contaminated open fractures.

Questions/purposes (1) Are rifamycin derivatives effective against established biofilms of clinical isolates of *S. aureus*? (2) Can PMMA be used as a carrier for rifamycin derivatives?

Methods Biofilms were developed and evaluated for susceptibility to a panel of antimicrobials in vitro using the minimum biofilm eradication concentration high-throughput model. Susceptibility was assessed by measuring bacterial recovery at 6 and 24 hours after antimicrobial treatment. Activity of rifamycin derivatives against intracellular bacteria was also evaluated using a gentamicin protection assay. Evaluation of PMMA as a carrier for rifampin and rifamycin derivatives was determined by assessing the curing time subsequent to loading of rifamycins and characterizing the release kinetics of rifamycins at daily intervals for 14 days from PMMA by performing bioassays.

Results Rifamycin derivatives between 1 and 8 μg/mL reduced bacteria within biofilms 5- to 9-logs and prevented bacterial recovery up to 24 hours post-treatment, indicating near to complete eradication of biofilms. Rifamycin derivatives at 32 μg/mL had activity against intracellular staphylococci, significantly reducing the number of internalized bacteria with limited effects on osteoblast viability.

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Rifampin was the only rifamycin observed to have a suitable release profile from PMMA, releasing 49% of the total antibiotic and maintaining a sustained released profile up to 14 days at a mean 28 ± 6 μg/mL.

Conclusions Rifampin can be incorporated into PMMA and eluted at concentrations effective against biofilms and intracellular staphylococci.

Clinical Relevance Our in vitro findings suggest that local delivery of rifampin may be an effective strategy for the prevention and/or treatment of open fractures where *S. aureus* biofilms might develop. Clinical studies are needed to characterize what role this approach might have in the prevention and treatment of infections involving biofilms.

Introduction

Extremity injuries account for the majority of wounds sustained by US service members in the current conflicts in Afghanistan and Iraq, with fractures accounting for up to 39% of these injuries. More than 80% of these fractures are classified as open, resulting from penetration of fragments secondary to a primary blast [43, 44]. Infection resulting from multidrug-resistant bacteria is a frequent complication, estimated to be between 39% and 64% of traumatic admissions with up to 27% developing persistent musculoskeletal infection, primarily resulting from methicillin-resistant *Staphylococcus aureus* (MRSA) [8, 9, 32]. Importantly, infections of open fractures continue to be a major source of patient morbidity often associated with increased rates of surgical revisions, time to osseous union, prolonged hospital course, extremity amputation, and reduced return-to-duty rates [5, 9, 34, 40, 53].

The management of high-energy orthopaedic wounds with resultant bone loss involves the collective administration of systemic antibiotics, débridement and irrigation of the wound, and temporary fixation with delayed definitive fixation [42, 58]. Local delivery, through antibiotic-impregnated poly(methylmethacrylate) (PMMA) beads, often aminoglycosides or glycopeptides, such as gentamicin or vancomycin, is used in combination with systemic antibiotics to achieve high local wound concentrations while preventing unwanted toxicity from systemic antibiotics [22, 37]. Although some studies have demonstrated a reduction in rates of infection with use of antibiotic-impregnated PMMA beads, including the use of vancomycin, there are other studies demonstrating a limited clinical benefit, which is in part thought to be the result of the persistence of bacteria within biofilms [4, 30, 35, 51, 58].

Biofilms are surface-attached communities of bacteria surrounded by a self-produced polymeric matrix displaying an increased resistance to antimicrobials making them difficult to eradicate [15, 17, 25, 48]. For orthopaedic infections, biofilms are recognized as an underlying cause of recalcitrance to antimicrobial therapies and an increase in infectious relapse as contributing to nonosseous union of open fractures [7, 14, 45, 50, 60]. Rifampin, a member of the rifamycin class predominantly used for treatment of tuberculosis, has been shown to have activity against staphylococcal biofilms [12, 21]. Rifampin primarily functions by inhibiting bacterial transcription; however, its excellent diffusive properties combined with activity that is independent of bacterial division allows for activity against biofilms [18, 59]. There are numerous in vitro, in vivo, and clinical studies that support the use of rifampin combination antimicrobial therapy for the treatment of staphylococcal bone and joint infections [6, 12, 19, 21, 23, 39]. Because of the rapid emergence of staphylococcal resistance to rifampin associated with monotherapy, combination antimicrobial therapies, including a nonrifamycin agent, have been recommended by the Infectious Diseases Society of America for bone and joint infections [35]. Although these studies support the use of adjunctive rifampin for use as a systemic treatment of staphylococcal infections, there are few studies to date evaluating the activity of other rifamycin derivatives against biofilms of *S. aureus* [41, 56]; moreover, it is unknown whether rifamycins are suitable to use with PMMA for local delivery for the treatment of contaminated open fractures [2].

To evaluate the potential application of rifampin and other rifamycin derivatives for local therapy of contaminated open fractures, the purpose of this study was to address the following: (1) Are rifampin and rifamycin derivatives effective against biofilms of clinical isolates of *S. aureus*? (2) Can PMMA be used as a carrier for rifamycin derivatives?

Materials and Methods

A convenience sample of seven genetically distinct clinical isolates of *S. aureus* from a strain repository collected from patients admitted to the San Antonio Military Medical Center (JBSA, Fort Sam Houston, TX, USA) as a part of treatment and not related to research and previously characterized for biofilm formation [1, 49] as well as two commercially available strains from the American Type Culture Collection (ATCC, Manassas, VA, USA) were used in this study (Supplemental Table 1 [Supplemental materials are available with the online version of CORR®.]). Isolates were cultured on blood agar plates (Remel, Lenexa, KS, USA) or in cation-adjusted Mueller-Hinton broth (MHB II) at 37 °C to an optical density of 0.1 at 600 nm (approximately 1.5×10^8 CFU/mL). The antimicrobial agents were purchased from Sigma-Aldrich (St

Louis, MO, USA) including cefazolin ciprofloxacin, clindamycin, oxacillin, trimethoprim-sulfamethoxazole, vancomycin, and the rifamycin derivatives rifampin, rifabutin, rifapentine, and rifaximin. Antimicrobial stocks were prepared according to recommendations and stored at −20 °C. Activities of antimicrobial agents were validated against Clinical and Laboratory Standards Institute (CLSI) reference quality control *S. aureus* strain (ATCC 29213) and were tested at a concentration range of 0.0625 to 128 μg/mL with the exception of sulfamethoxazole, which was tested at a concentration range of 0.593 to 2432 μg/mL.

Biofilms were developed and evaluated for susceptibility to antimicrobial agents using minimum biofilm eradication concentration (MBEC) P&G plates (Innovotech, Alberta, Canada) as previously described with some minor modifications (Supplemental Fig. 1 [Supplemental materials are available with the online version of CORR®.]) [11, 13, 39]. Briefly, bacteria (1×10^6 CFU/mL) were added to individual wells of the MBEC plates and incubated overnight at 37 °C with shaking at 150 rpm (VWR, Radnor, PA, USA). After incubation, the plate tops containing the pegs with biofilms were rinsed in phosphate-buffered saline (PBS), placed in a challenge plate containing the antimicrobial agents, and incubated for an additional 24 hours. Pegs were rinsed and sonicated for 15 minutes at 40 kHz (Branson Ultrasonics Corp, Danbury, CT, USA) into a 96-well plate containing MHB II for bacterial recovery. Bacterial viability was determined by plating 10-μL serial dilutions onto MHB agar at 6 and 24 hours after recovery. Assays were performed in triplicate. As a qualitative analysis, scanning electron microscopy (SEM) of biofilms grown on pegs treated as described previously and allowed to recover for 24 hours was performed. Representative SEM images were taken using a JEOL-6610 scanning electron microscope (JEOL Inc, Peabody, MA, USA).

Cytotoxic effects of rifamycins on human osteoblasts (PromoCell, Heidelberg, Germany) were measured using the XTT Cell Proliferation Assay (ATCC, Manassas, VA, USA). In brief, osteoblasts, maintained in Minimum Essential Medium, supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (Invitrogen, Grand Island, NY, USA), were seeded into 96-well plates at 1×10^5 cells/well and treated with rifamycins diluted in unsupplemented Gibco® StemPro® MSC SFM XenoFree cell media (Life Technologies, Grand Island, NY, USA) for 24 hours at 37 °C in 5% CO_2. Cells were then rinsed with PBS and media was replaced with a 1:1 volume of fresh StemPro® media and XTT Cell Proliferation Assay mix (ATCC) and incubated for 3 hours. Absorbance was measured at 475 nm and toxicity of data was reported as percent cell viability compared with cell viability of untreated cells. Assays were performed in triplicate.

Intracellular invasion assays were performed to evaluate the effectiveness of rifamycins against internalized *S. aureus*, as previously described [33]. Briefly, osteoblasts were seeded in 24-well plates at 2×10^4 cells/mL and infected with *S. aureus* at a concentration corresponding to a multiplicity of infection of 50. Cells were incubated at 4 °C for 30 minutes allowing for bacterial sedimentation and at 37 °C for 1 hour for invasion. Cells were then rinsed with PBS, treated with 200 μg/mL gentamicin to eliminate extracellular bacteria for 1 hour followed by exposure to media containing vancomycin or the rifamycin derivatives for up to 24 hours. At 4 and 24 hours, the number of viable, intracellular bacteria was determined by plating serial dilutions of cell lysates onto blood agar. As a qualitative, visual assessment of the activity of rifamycins, fluorescent microscopy was performed. Briefly, cells were seeded and infected as described previously followed by fixing in 3.7% formaldehyde and staining with a fluorescein isothiocyanate (FITC)-labeled anti-*S. aureus* antibody (Cat #AB20920; Abcam, Cambridge, MA, USA), an Alexa Fluor® 577 Phalloidin F-Actin stain, and NucBlue® (Life Technologies). Images were captured at × 40 using an Olympus IX71 inverted fluorescence microscope (Olympus America, Center Valley, PA, USA).

To evaluate PMMA as a carrier for rifamycin and the rifamycin derivatives, we assessed the following properties: curing time of PMMA loaded with rifamycin antimicrobials and the release kinetics of rifamycins from PMMA by performing bioassays. PMMA beads with 10% antibiotic were made by combining 40 g PALACOS® bone cement powder (Zimmer Orthopaedic Surgical Products, Dover, OH, USA) with 6 g rifamycin or vancomycin powder. Methylmethacrylate monomer (20 mL) was added to the powder, mixed thoroughly, and spread across a 3-mm mold, creating beads weighing approximately 20 mg each. To measure elution concentration and activity of rifamycin, PMMA beads (three/group) were placed into 2 mL PBS and incubated at 37 °C. Eluents were removed and replenished with fresh PBS daily for up to 14 days. Antibiotic concentration was determined by correlating the zone of inhibition of the eluents with zones from known concentrations of antibiotics. Mueller Hinton plates were inoculated with a CLSI reference quality control *S. aureus* strain (ATCC 29213). Paper discs (6 mm diameter) were loaded each with 20 μL of eluent or antibiotic of known concentration. The discs were placed onto the prepared plates and incubated overnight.

Statistical analysis of the data from the bacterial invasion was performed using a one-way analysis of variance with Dunnett's posttest for comparison between groups using GraphPad Prism 5 software (San Diego, CA, USA) with p values of 0.05 considered to be statistically significant.

Results

With the exception of rifamycins, other commonly used antimicrobial agents to treat staphylococcal infections, to which the clinical isolates were susceptible in the planktonic form (Supplemental Table 1), were observed to have limited activity against established biofilms of methicillin-resistant and methicillin-susceptible isolates, reducing but not promoting complete eradication of viable bacteria within the biofilms (Table 1). Treatment of biofilms with ciprofloxacin, clindamycin, trimethoprim/sulfamethoxazole, and vancomycin (for MRSA/methicillin-sensitive *S. aureus* [MSSA]); cefazolin and oxacillin (for MSSA), at ranges between 0.0625 and 2 μg/mL, resulted in the reduction of viable bacteria within biofilms from 4- to 8-log compared with untreated controls, effectively hindering the recovery of viable organisms up to 6 hours, but not after 24 hours where no considerable loss in bacterial viability was observed after treatment (Table 1; Supplemental Tables 2, 3 [Supplemental materials are available with the online version of CORR®.]). In contrast, rifamycins including rifampin, rifabutin, rifapentine, and rifaximin, at ranges between 1 and 8 μg/mL, reduced viable bacteria within biofilms from 5- to 9-logs and prevented bacterial recovery rate up to 24 hours post-treatment, indicating near to complete eradication of biofilms (Table 1; Supplemental Tables 2, 3). Consistent with this, qualitative analysis of biofilms using SEM confirmed activities of the rifamycin derivatives, demonstrating near eradication of viable bacteria and little to no biofilms present on the pegs after 24 hours recovery posttreatment (Fig. 1). In addition to the activity against biofilms, rifampin and the rifamycin derivatives at a concentration of 32 μg/mL were able to reduce the number of intracellular *S. aureus*, an important source of organisms contributing to persistent and recurrent infections [26, 55] compared with vancomycin at 4 and 24 hours after infection (Figs. 2A, 2C). Notably, rifampin and the rifamycin derivatives at concentrations $\geq$ 64 μg/mL showed cytotoxic effects on uninfected osteoblasts in the XTT cell growth assay as $<$ 60% of cells were viable after 24 hours of treatment (Fig. 2B).

Incorporation of the rifamycin derivatives into the PMMA increased the time required for complete curing of PMMA beads to approximately 1 hour compared with approximately 15 minutes for the curing with vancomycin. Each antibiotic evaluated produced a unique release profile with the lowest detectable ranges using the bioassay to be between 0.25 and 8 μg/mL. Vancomycin had a large initial burst releasing 15% of its load within the first 3 hours of incubation and 28% of its load within the first 24 hours reaching mean concentrations of 494 ± 118 and 338 ± 130 μg/mL (Fig. 3A). After the initial burst, release of vancomycin was much slower and sustained for up to 4 days with detectable levels up to 10 days. Rifampin also had an initial burst releasing 5% and 14% of load over the first 3 hours and 24 hours, respectively (Fig. 3B). Notably, rifampin had a sustained-release profile from PMMA up to the 14 days evaluated, eluting at a mean of 28 ± 6 μg/mL per day. By Day 14, the rifampin-loaded beads released 49% of their antibiotic loading compared with the 39% eluted from the vancomycin beads at Day 10. Rifabutin, rifapentine, and rifaximin released much lower concentrations with only 0.5%, 0.73%, and 0.6% of total load being released from the PMMA beads after 24 hours and only 0.63%, 1.5%, and 2% after 7 days, respectively. Elution of rifabutin and rifaximin fell below detectable amounts by Day 7, whereas rifabutin and rifapentine, although slow, continued to elute at rates averaging 3 to 4 μg/mL per day. On inspection of the bacterial plates, it was obvious that the concentrations of the elutions decreased over time in the rifaximin, rifabutin, and vancomycin groups. The zone of inhibition was its largest at Day 1 with smaller diameters at Days 7 and 14 for rifampin and rifapentine (Fig. 3C).

Discussion

Infectious complications of open fractures are a major source of morbidity among US service members [8, 9, 43, 44]. Historically, implantation of antibiotic-impregnated PMMA within the wound has been used to help reduce bacterial load. However, the effectiveness of treatment may be limited for a number of reasons including the development of antimicrobial resistance and/or the limited activity against persistent bacteria within biofilms to the chosen antibiotic [4, 20, 30, 50]. Although rifampin has been documented to have activity against staphylococcal biofilms in vitro and has been indicated for use as an oral or intravenous combination therapy for the treatment of staphylococcal osteoarticular infections [12, 21], few studies have evaluated the potential use of rifampin or rifamycin derivatives addressing local delivery of antimicrobials for treatment of contaminated open fractures [28, 29, 38]. We demonstrate that rifampin and rifamycin derivatives display activity against staphylococcal biofilms as well as internalized bacteria within osteoblast compared with other commonly used agents and, moreover, that rifampin can be loaded and eluted from PMMA beads successfully, despite previous literature suggesting otherwise [16, 27]. These in vitro results indicate that for orthopaedic infections involving *S. aureus* biofilms, rifampin may be an applicable alternative to currently used agents in orthopaedic and trauma surgery.

Our present study does have some limitations. First, the in vitro model used for biofilm development and susceptibility testing cannot adequately recapitulate biofilms developed in vivo, in which host factors absent under

Table 1. Activities of antimicrobial agents against biofilms of clinical isolates of *Staphylococcus aureus*

Antimicrobial agent	MRSA (n = 5)				MSSA (n = 3)			
	6 hours		24 hours		6 hours		24 hours	
	MBIC (range)*	Log reduction†	MBIC (range)	Log reduction	MBIC (range)	Log reduction	MBIC (range)	Log reduction
Ansamycin								
Rifampin	0.0625 (0.0625–0.5)	6.3 ± 1.8	8 (1–32)	5.0 ± 4.8	0.0625 (0.0625–0.125)	5.19 ± 1.6	8 (1–16)	5.2 ± 3.4
Rifabutin	0.0625 (0.0625–0.5)	5.2 ± 2.2	1 (0.0625–2)	5.1 ± 3.8	0.0625 (0.0625–0.125)	8.1 ± 1.0	0.25 (0.0625–0.25)	7.5 ± 5.8
Rifapentine	0.0625 (0.0625–0.5)	7.9 ± 2.0	0.5 (0.125–2)	8.0 ± 3.0	0.0625	8.1 ± 1.0	0.25 (0.125–1)	9.7 ± 2.9
Rifaximin	0.0625 (0.0625–0.25)	5.2 ± 0.9	4 (1–8)	9.6 ± 3.2	0.0625 (0.0625–0.125)	6.6 ± 3.2	2 (1–4)	9.8 ± 3.0
Cephalosporin								
Cefazolin	> 128 (4 to > 128)	2.5 ± 4.2	> 128	0.0 ± 2.8	0.0625 (0.0625–0.125)	4.6 ± 1.6	> 128	2.4 ± 3.4
Fluoroquinolone								
Ciprofloxacin	0.5 (0.0625 to > 128)	4.6 ± 3.2	> 128	3.1 ± 3.0	0.0625 (0.625–0.125)	6.9 ± 1.2	> 128	0.2 ± 2.4
Glycopeptide								
Vancomycin	1 (0.5–8)	5.1 ± 2.1	> 128	0.8 ± 2.8	0.5 (0.5–1)	5.0 ± 2.1	> 128	0.0 ± 0.1
Lincosamide								
Clindamycin	0.5 (0.25 to > 128)	4.4 ± 2.4	> 128	2.6 ± 5.5	0.0625 (0.0625–1)	6.5 ± 3.7	> 128	1.9 ± 3.1
Penicillin								
Oxacillin	> 128 (2 to > 128)	3.6 ± 3.9	> 128	0.8 ± 3.1	0.0625 (0.0625–0.125)	6.0 ± 2.3	> 128	3.3 ± 3.0
Sulfonamide								
Trimethoprim/ sulfamethoxazole	0.0625/1.19 (0.0625/1.19–0.5/9.52)	5.8 ± 2.2	> 128/2437	3.3 ± 2.9	0.0625/1.19 (0.0625/1.19–0.125/238	5.5 ± 1.8	> 128/2437	0.3 ± 2.3

* Minimal biofilm inhibitory concentration (MBIC) indicates the median (range) concentration of antibiotic (μg/mL) that resulted in inhibition of the biofilm as indicated by the reduction of viable bacteria ≥ 50% of the control untreated groups (media only) after 6 and 24 hours of bacterial recovery after overnight treatment of the isolates of methicillin-resistant and methicillin-susceptible (MRSA/MSSA) *S. aureus*; †log reduction; indicates the corresponding mean (Log_{10}CFU/mL) ± SD reduction of viable bacteria within biofilms of MRSA/MSSA isolates.

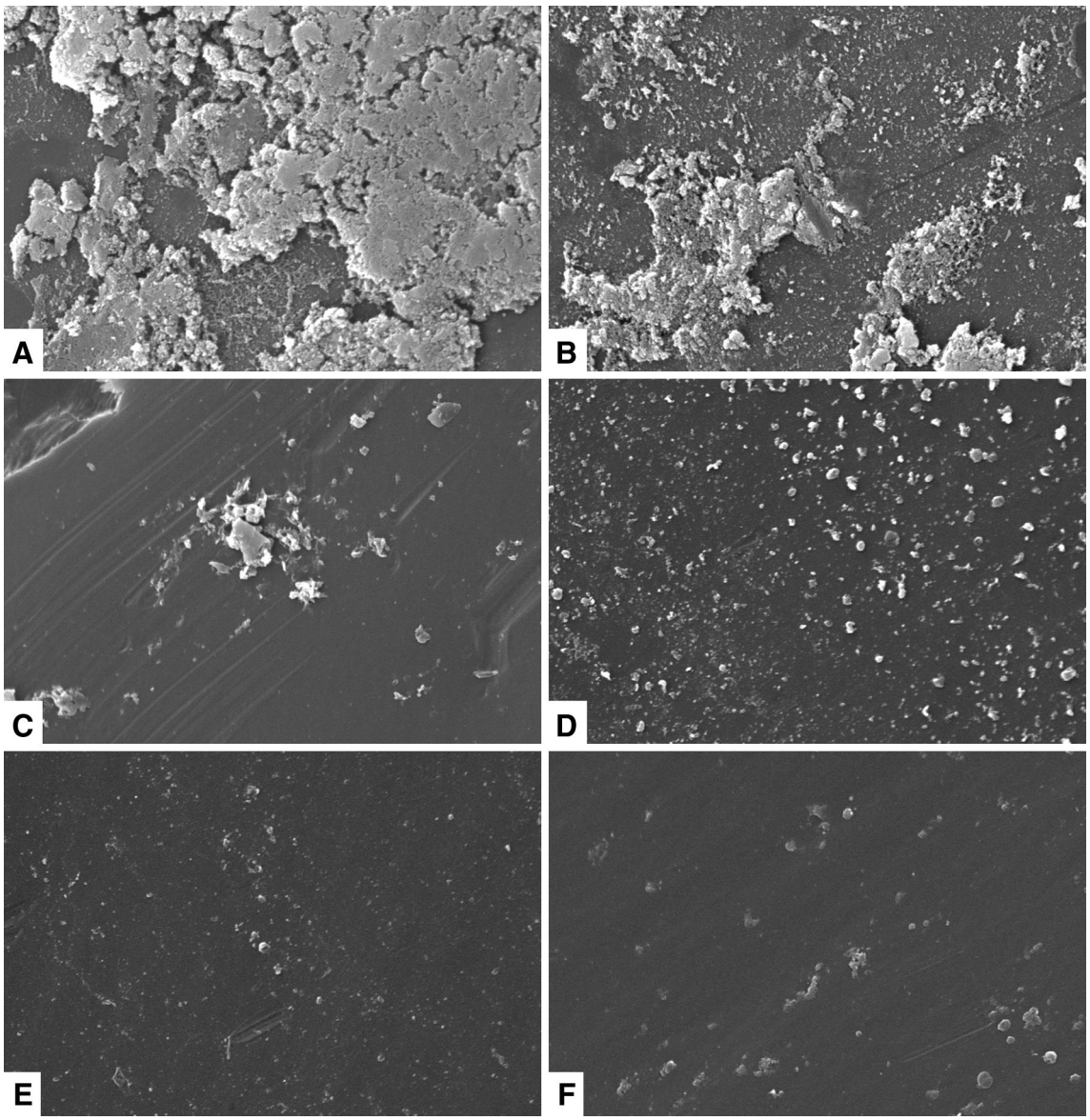

Fig. 1A–F Representative SEM images show biofilms of *S. aureus* strain SAMMC-58 on individual pegs of the MBEC P&G device after 24-hour recovery in antibiotic-free media with no treatment, (**A**) control, or after overnight antimicrobial treatment with (**B**) vancomycin (128 μg/mL), (**C**) rifampin (32 μg/mL), (**D**) rifabutin (2 μg/mL), (**E**) rifapentine (4 μg/mL), or (**F**) rifaximin (8 μg/mL) (× 1500).

in vitro culture conditions could influence the biofilm phenotype and possibly affect antimicrobial activities observed herein [10]. However, the biofilm model used in this study is well characterized and has been demonstrated to be a reliable, reproducible experimental tool for evaluating susceptibilities of bacterial biofilms to various agents [3, 11, 13]. Importantly, the ruggedness (ie, insensitivity) of the assay to small changes in the protocol [46] allows investigators to use these models to evaluate treatments independent of laboratory conditions, making it a useful model for susceptibility testing.

Additional limitations of our study were the evaluation of only PMMA as a carrier and the use of rifamycins as a monotherapy. Antibiotic-loaded PMMA cement spacers or beads are commonly used as strategies for managing infection control and to minimize cavitary soft tissue defects providing temporary length stability of bony defects within orthopaedic injuries requiring multiple revision surgeries. PMMA was evaluated as the carrier in this study given its applicability for clinical use in prophylaxis and/or treatment of osteomyelitis and, moreover, to assess whether rifamycins were compatible for incorporation and use with

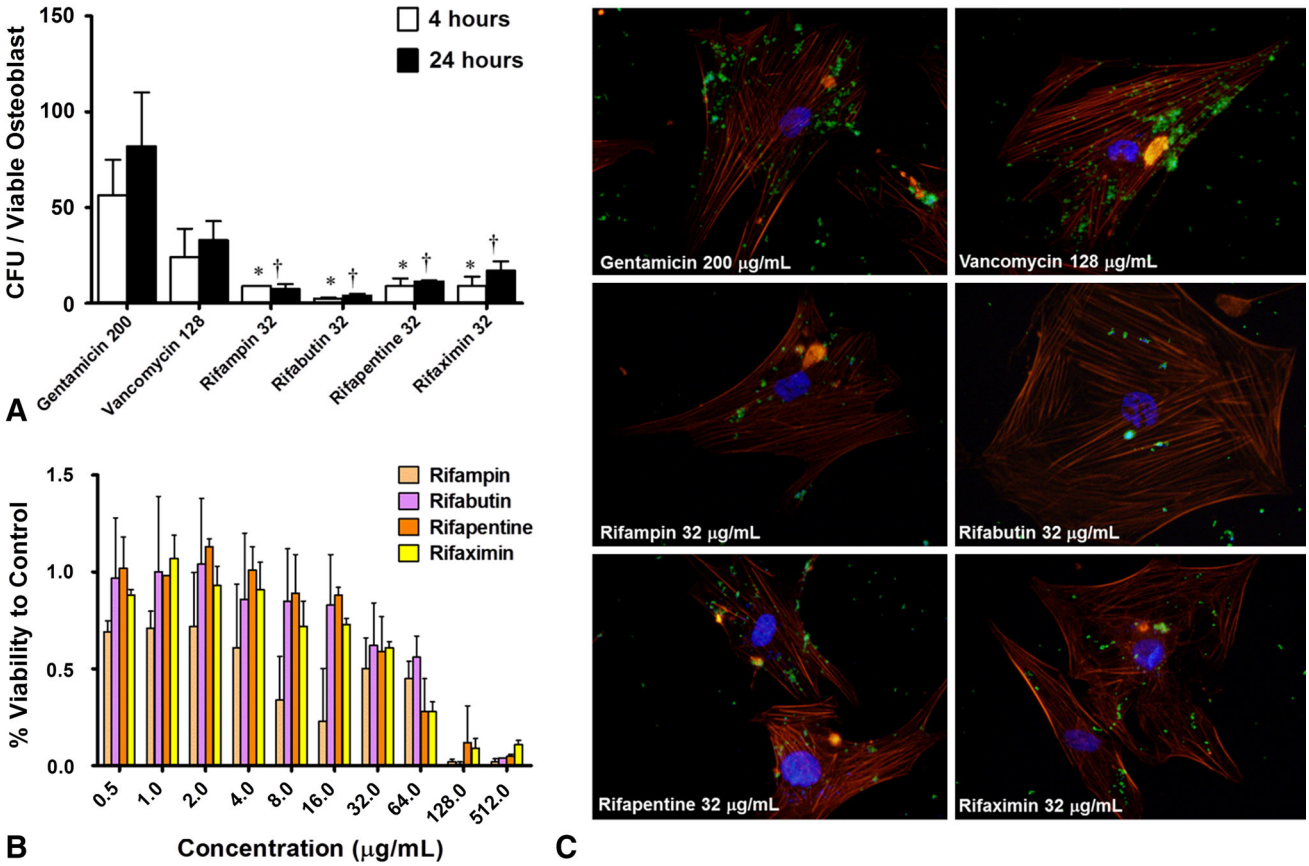

Fig. 2A–C Effects of rifampin and rifamycin derivatives on intracellular survival of *S. aureus* strain SAMMC-58 in human osteoblasts are shown. (**A**) Viable intracellular bacteria after 4 hours and 24 hours of treatment with gentamicin (200 μg/mL), vancomycin (128 μg/mL), rifampin, or the rifamycin derivatives (32 μg/mL) are shown. Values are reported as the mean ± SD of n = 6 samples. *,† Significant differences compared with vancomycin ($p < 0.05$) at 4 and 24 hours, respectively, as determined by one-way analysis of variance. (**B**) Percent cell viability of osteoblasts treated with increasing concentrations of rifamycin and rifamycin derivatives to cell viability of untreated cells is shown. Values are reported as the mean ± SD of n = 3 samples. (**C**) Representative immunofluorescence microscopy images show viable, intracellular bacteria after 24 hours treatment with antibiotics (green; FITC-labeled anti-*S. aureus* antibody, red; Phallodin F-Actin stain, blue; nuclear stain; original magnification × 400).

this platform. Notably, the heat-liable nature of certain antimicrobial agents makes them unable to withstand the exothermic polymerization reaction that occurs with PMMA cement; conversely, the antimicrobial agent can potentially impair the polymerization of PMMA and this can limit its use as a carrier. Furthermore, although absorbable mineral-based bone cements such as calcium phosphates and calcium sulfate provide advantages to PMMA beads as a result of their improved release kinetics, bioabsorbable properties, and their compatibility with a wider range of antibiotics [36, 54], it is important to note that these carriers may be most appropriate for procedures in which a return to the operating room is not anticipated as a result of their relative cost. Future studies evaluating the incorporation and release of rifamycins from other carriers such as bioabsorbable bone cements and graft substitutes for use at the time of definitive management are necessary to extend application of this intervention. Lastly, in our study, we demonstrated that rifampin and other rifamycin derivatives were effective as a monotherapy against biofilms of clinical isolates of *S. aureus* in vitro. In contrast, clinical application of rifampin would require it to be used with a second nonrifampin agent, rather than a monotherapy, to reduce incidence of the emergence of resistance [12, 35] administered concomitantly systemically or codelivered directly.

Antimicrobial agents to be incorporated into PMMA should be appropriate for the treatment of organism(s) suspected of causing infection. The ability of *S. aureus* to persist within biofilms makes the treatment of orthopaedic infections difficult [14, 50]. The majority of currently available antimicrobial agents, including vancomycin, primarily act to inhibit replication of planktonic bacteria and thus have shortcomings as treatments for

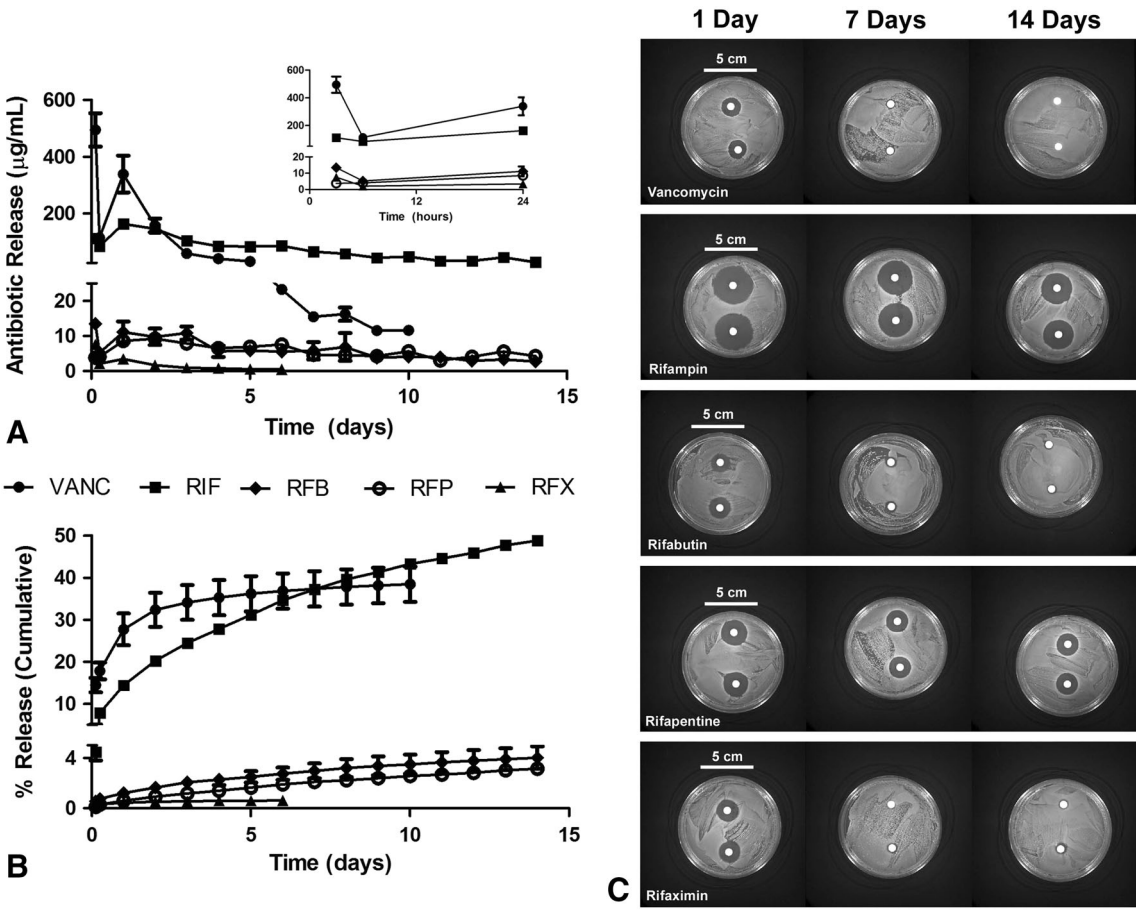

Fig. 3A–C Release of vancomycin (VANC), rifampin (RIF), rifabutin (RFB), rifapentine (RFP), and rifaximin (RFX) from PMMA beads, loaded at a 10% w/w, over 14 days is shown. (**A**) Antimicrobial release (μg/mL) from PMMA was determined by comparing the mean size of zone of inhibition of eluents recovered to zones of inhibition created using antibiotic standards at known concentrations (graph inset represents antimicrobial release from PMMA during the first 24 hours). (**B**) Cumulative release of the total antimicrobial loaded from PMMA beads is shown. Values are reported as the mean ± SD of n = 3 samples. (**C**) Representative images shows the zones of inhibition from collected eluents of the various antimicrobial-loaded PMMA beads at 1, 7, and 14 days. Release of rifamycins from PMMA beads over 14 days is shown.

biofilm bacteria, in part given their metabolically inert, nonreplicating state. In our susceptibility testing, evaluation of bacterial recovery at 6 and 24 hours allowed for us to identify antimicrobials capable of inhibiting and/or eradicating (ie, significantly reducing) viable bacteria embedded within the biofilm. Consistent with other studies evaluating the effects of antimicrobials against biofilms of *S. aureus* [21], the majority of antibiotics tested were not able to effectively reduce bacteria embedded within the biofilms as indicated by high rates of recovery after 24 hours. In contrast, rifampin and the rifamycin derivatives were observed to have the most notable effect against biofilms of clinical isolates of *S. aureus*, significantly decreasing bacterial cell recovery. The ability of rifampin to readily diffuse and its activity independent of bacterial growth make it an ideal treatment for biofilms. Although this is not the first report documenting activity of rifampin against biofilms, our study confirms and extends these observations to other rifamycin derivatives, including rifabutin, rifapentine, and rifaximin, which to our knowledge have not been previously evaluated for activity against staphylococcal biofilms [24, 47, 52]. Studies demonstrating the role of biofilms in orthopaedic infections highlight that rifamycin class agents may be an effective antimicrobial agent for the clinical management of infection.

The ability of *S. aureus* to invade osteoblasts has been cited in addition to biofilm formation as a major mechanism of persistence and pathogenic event contributing to bone loss during infection [20, 26]. Internalized staphylococci avoid the host immune responses as well as the action of many forms of antibiotics making them difficult to eradicate similar to biofilms. Consequently, the intracellular activity of antimicrobial agent is a factor to consider when selecting an agent for clinical use. Rifampin and the

rifamycin derivatives were observed to significantly reduce the number of viable intracellular staphylococci with human osteoblasts at concentrations with limited toxicity further adding to the potential usefulness of this class of antibiotic agents for treatment of open fractures.

Antimicrobials loaded into PMMA must also elute at adequate local concentrations, often above minimum inhibitory concentration values, and have a sustained release lasting the course of therapy. We were able to show that several rifamycins could be loaded into PMMA. Rifampin eluted consistently, whereas rifabutin and rifapentine eluted at a much lower rate over a 14-day period. In contrast, rifaximin did not elute at detectable limits past the first 24 hours. From this, it can be determined that the specific antibiotic can affect the specific release profile, which influences the antibiotic of choice for clinicians. Just as Anguita-Alonso et al. and others demonstrated [2, 16, 27], we noticed that adding the rifamycins slowed the curing of the PMMA beads. This may potentially limit the use of rifamycin-impregnated PMMA beads prepared at the time of surgery in the operating room as a result of the time constraint. Despite these limitations, future studies addressing optimized concentrations to use for curing while maintaining the desired release characteristics may make this treatment approach clinically feasible.

Rifampin has been used clinically for decades but its systemic toxicity appears to limit its usefulness against established biofilms. Likewise, PMMA beads have been historically used to deliver high levels of antibiotics in the wound, but its lackluster reported effectiveness is likely the result of the fact that the antibiotics that are commonly delivered are not effective against the cause of infection, which may be the result of bacteria within biofilms [28, 29, 31, 57]. In this in vitro study, we demonstrated the rifamycin derivatives, compared with other commonly used antimicrobials to treat staphylococcal bone infections, were effective against both established biofilms and intracellular bacteria, two critical aspects relevant to disease pathology caused by this organism in orthopaedic infections. Of the rifamycin derivatives, only rifampin eluted from PMMA beads in a favorable manner because the others mostly stayed sequestered within the beads. The rifampin delayed the curing of the PMMA beads, which could reduce its clinical usefulness. Future studies will evaluate different currently available local carriers to address this concern. Changing the percentage of rifampin and combining with other antibiotics may also speed the curing of the PMMA.

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